Millet Response to Root Inoculation with Putative Growth Promoting Rhizobacteria Isolated from a Sahelien Shrub

Research Thesis

Hydrologic Redistribution and Rhizosphere Biology of Resource Islands in Degraded Agroecosystems of the Sahel: A PIRE in Tropical Microbial Ecology

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ABSTRACT

In agriculture systems like the Sahel of West Africa, where climatic and anthropomorphic events have degraded the soil, the reduced crop yield can severely impact the economy of the country and the livelihood of its citizens. Furthermore, most farmers operate at the subsistence level and cannot afford chemical fertilizers to increase crop production. However, two common shrubs *Guiera senegalensis* and *Piliostigma reticulatum*, commonly found in farmers' fields of the Sahel hold potential as local and low cost resource for improving crop yield in millet and peanuts. *G. senegalensis* performs hydraulic lift which moves water from the wet subsoil to the dry surface soil at night when photosynthesis stops. This hydraulically lifted water could support microorganism even in the dry season and may assist crops through drought periods. Crop yields are higher in the presence of shrubs, but the mechanism for this response is not well understood. We hypothesized that shrubs may harbor a diverse community of Plant Growth Promoting Rhizobacteria (PGPRs). Therefore, the objective was to measure the growth response of millet in presence and absence of inoculating with putative PGPR from the genus *Bacillus* in a one-month greenhouse experiment in Dakar, Senegal. Using amplified ribosomal DNA restriction analysis (ARDRA), 11 different groups were identified in rhizosphere soil taken from *G. senegalensis* in farmers' fields. The millet plants were inoculated with individuals from each group, and the plants' growth measured. Using pair-wise comparisons between the control groups, water and bacterial medium, it was found that one of the *Bacillus* groups significantly ($P < 0.1$) increased the growth of millet plants.

INTRODUCTION

Agroforestry systems in which trees or shrubs grow in fields with crops can increase inputs of organic matter and improve nutrient use and efficiency, as well as potentially harbor a

more diverse microbial population. This population includes plant growth promoting rhizobacteria (PGPRs). These organisms provide various benefits to plants, such as suppression and antagonism of pathogens, the induction of systemic disease resistance in plants, production of plant growth hormones, Nitrogen fixation, and solubilization of Phosphorous.

Rhizobacteria are micro-organisms that inhabit the inside of root cells between cell walls and the rhizosphere, the area of soil immediately surrounding the plant's roots including, respectively. The small zone of soil surrounding the roots receives carbon (C) and nitrogen (N) exudates, which promote bacterial growth in the rhizosphere (Lugtenberg 2009, Dossa et al 2008). Knowledge of the ecological functions of microorganisms play in root colonization is crucial to developing biological controls of soil-borne pathogens and the effective use of beneficial microorganisms to enhance crop yield (Kloepper et al 1980). Since the rhizosphere microflora are extremely diverse, a dynamic interplay between the members of the microbial community occurs, which is heavily influenced by other organisms present in the system as well as nutrient limitations (Garbeva et al 2004, cited in Kumar et al 2011).

Bacteria exhibiting properties common to PGPRs include genera *Bacillus, Pseudomonas, and Actinobacteria*. *Bacillus* is one of the most well-known and studied genera of PGPR organisms because of its abundance in many environments and that is may have many beneficial physiological traits such as a stress-resistant endospore formation, a multi-layered cell wall, and extracellular enzymes. *Bacillus* or other PGPRs may provide biocontrol of disease and ac as biofertilizers in soil systems. Mechanisms of biocontrol include production of antibioics, secretion of lytic enzymes, and induction of Systemic Resistance (ISR) in the host plant. Examples of biofertilization include nitrogen fixation, inorganic phosphorous solubilization, and production of siderophores (Kumar et al 2011). Additionally, the use of PGPRs is attractive

because the use (or overuse) of chemical herbicides, pesticides and fertilizers are causing health and environmental concerns (Kumar et al 2011).

Biocontrol

A recent trend in research is to focus on the use of plant growth promoting rhizobacteria to suppress soil-borne pathogens through several mechanisms including the production of nutrients and stress reduction through manipulation of phytohormones in root exudates and the surrounding soil (Yang et al 2012). *Bacillus* and other microorganisms play a key role in this by maintaining plant health through the production of auxins such as Indole-3-acetic acids (IAAs), cytokinins, ethylene, or gibberellins, in the rhizospheres of their hosts. Both are phytohormones, which when produced by bacteria, have been shown to effectively increase plant height. However, several factors affect the contribution of IAAs, namely the concentration of IAA-producing bacteria in a sample and their individual abilities to produce IAAs (Loper and Schroth 1986). A study by Mena-Voilante and Olalde-Portugal, 2007, verifies that the inoculation of tomato plants *Bacillus spp*., resulted in a statistically significant increase in dry root biomass of 18 - 26% and length increase of 13–15% compared to a control group in a greenhouse study. Similarly, the inoculated tomato plants showed an increase of biomass of 21% and fresh fruit weight increase of 20%, which included a significant increase in the yield of plants suitable for the general market (Mena-Voilante and Olalde-Portugal 2007).

Additionally, it was found in a study by Loper and Schroth in 1986 that many species increased the shoot: root ratio in their host even when present in low concentrations, and in high concentrations these species produced enough IAAs to significantly increase the root length of their sugar beet host. These and other phytohormones produced in the soil can be transported into the plant tissues by epiphytic bacteria to raise endogenous hormone levels and stimulate growth.

Other species were found to significantly inhibit the growth of a competing plant in the area or inhibit the growth of the host species when inoculated in concentrations above the threshold dose of 10^{-6} to 10^{-9} mols(L^{-1}) by severely disrupting the hormone balance in the system (Loper and Schroth 1986). This may have indirect effects on the microbial community the plant harbors, although at the time of this study, more research was necessary (Loper and Schroth 1986).

Through the production of phytohomones and other chemicals, many of these species may also be antagonistic to soil pathogens and may also stimulate systemic resistance systems of their host plant to protect it from foliar and root pathogens. The generally non-specific character of induced resistance may increase resistance to several pathogens simultaneously, which is of benefit in the presence of multiple pathogens (Van Loon 2005). ISR is similar to Systemic Acquired Resistance (SAR) in that both are induced forms of resistance in which the plant has been preconditioned to ward off a broad range of pathogens, however, the genes "primed" to will be activated in larger concentrations in plants with SAR than in plants with ISR. These kinds of resistance differ in the nature of their elicitor and the pathway involved. For example, SAR is triggered by exposing a plant to virulent, avirulent, or nonpathogenic microbes, which stimulate the production of antipathogenic proteins such as chitinase and salicyclic acid (SA) in the rhizosphere. When challenged by a pathogen, plants with SAR will express defense-related genes responding to concentrations of SA in the soil. Similarly, ISR depends on non-pathogenic bacteria (PGPRs) operating in a system regulated by jasmonic acid and ethylene signaling from the plant. The ISR pathway is initiated when the plant is attacked by a pathogenic microorganism and sends out jasmonic acid as a signaling protein, creating a cascade of antifungal and oxidative enzymes that remove the pathogen.

However, it has been discovered that there is a third pathway for ISR in plants: Rhizosphere-Mediated Induced Systemic Resistance (RISR). The pathway is elicited by the production of jasmonic acid or ethylene as in ISR, but the leaves of these plants exhibit increased amounts of salicylic acid, as does SAR. Several *Pseudomonas* and *Bacillus* strains have been found to induce systemic resistance in a wide range of crops, and some strains exhibit specificity for particular crops. Although the elicitors of RISR are difficult to determine, it is thought that the pathway includes the use of flagella, siderophore, and O-antigenic lipopolysaccarhides or other components common to both the host and PGPR (Van Loon 2005). In a study by Chourday et al (2008), protein transcription analysis was conducted on a PGPR strain of *Pseudomonas* and its host plant *Arabidopsis*, and it was found that in leaves and roots, the genes required for a systemic response were only found in significant amounts after an attack by a necrotizing pathogen, implying that the plant had signaled for the aid of the bacteria. The ISR system took over at this point to spread the effects of the RISR to the rest of the plant. The combination of ISR and RISR is seen in other systems as well, including the above- and belowground systems of fruit bearing trees. Although the effectiveness of both kinds of resistance varies from species to species, all plants will be more able to defend against pathogens when they exhibit all resistance pathways (Chourday et al 2008).

The induction of systemic resistance of their host can be mediated by the production of antibiotics, protecting them against an even wider range of pathogens. In particular, *Bacillus sp.* are known to produce Kanosamine, Zwittermycin A, Iturin A (Cyclopeptide), Bacillomycin, and Plipastatins A and B, which may posses antimicrobial, antiviral, anti-herbivory, anti-helmenthic, phytotoxic, cytotoxic, or growth promoting properties (Fernando et al 2005). Of these antibiotics, several have been more extensively investigated, which include a group of sterol dependent

antifungal, Zwittermycin and Bacillomycin, which are variants of Iturn A and Sufractin. Iturin A and Surfactin possess weak to moderate antibiotic factors, but are powerful surfactants and strongly amphiphilic. In a study by Asaka and Shoda, 1996, it was found that *Bacillus subtillus* RB14-C produces these chemicals as a safe and effective biological disease control agent of damping off in tomato seedlings. They observed an 84% decrease in dry biomass in plants inoculated with only the pathogen *Rhizoctonia solani* when compared to tomato seedlings inoculated with *B. subtillus* RB14-C and the pathogen. It was observed that 11% of diseased plants recovered when inoculated with this strain, and that the strain could be used as a soil additive in both its motile and cyst forms (Asaka and Shoda, 1996). This indicates that this particular PGPR may remain active in various soil conditions, which would be useful for agricultural applications.

Although *B. subtillus* is fairly resistant to changes in soil conditions, many factors may affect the production and efficacy of PGPR antibiotics including physical properties of the soil, nutrient level, level of acetylation, cell growth stage and density, and variable sensitivity within and between microbial populations (Fernando et al 2005). Unfortunately, the complexity of biotic and abiotic interactions affects the recovery and detection of these antibiotics. These interactions are not limited to nutrient content in the soil, but also include irreversible binding to soil particles, effects on other members of the microbial community, and chemical degradation of the compounds (Thomashow et al 1997; cited in Fernando et al 2005). This has restricted, but also inspired, further research to better understand potential biocontrol agents in agriculture, and their limitations. For example, however well an antibiotic may work to eliminate a pathogen in vitro, in vivo studies have found that the concentrations of antibiotics may be too low to inhibit a plant pathogen, and that the interactions of low concentrations of antibiotics and other

extracellular metabolites may simply trigger SAR in the host (Fernando et al 2005).

Biofertilization

PGPRs also function in soil systems to increase the amount of nutrients available to plants. Nitrogen is one such nutrient, and is made available to plants through the biological processes of mineraliztion and fixation of N_2 from the atmosphere (Johnson et al 2005). One of the most important functions of PGPRs in agriculture, N fixation is restricted to prokaryotes and is catalyzed by the nitrogenase complex. Agricultural practices tend to remove more N from soils than can be readily replaced, limiting crop production generally, necessitating the excessive addition of N fertilizers. Frequently, chemical fertilizers can have negative effects on the environment. Consequently, PGPRs, particularly the genus *Paenebacillus*, are being investigated as a potential, cost-effective way to increase N inputs in agricultural systems and reducing the need for chemical inputs (Van Berkham, Bohool 1980). The genus *Paenebacillus* contains some of the most efficient N fixing bacteria studied, so far. *P. durus* (formerly *P. azotofixans*) is commonly found to fix N in systems containing wheat, maize, or banana crops in tropical regions (Rosado et al 1998). This species, like several others of the same function, is found to contain the nitrogenase reductase nifH gene in its genome. The nif operon, which controls the use of this gene, is expressed in the presence of N and converts it from the unusable N_2 form to the NH_3 form in microbial biomass. This can be absorbed by the plant's roots and used in the production of its nucleotides and other amino acids (Tiexera et al 2008).

Phosphorous (P) is another essential, yet limited, nutrient sometimes present in soil systems in just millimolar concentrations (Ozanne 1980, Goldstein 1994; cited in Fankam et al 2006). In plants, Phosphate is essential for the production of ATP and DNA, as well as cell growth and development, even though it comprises only 2% of their weight. Because of this,

decreased levels of P in soils can lead to delayed maturation of crops, low-yield, and decreased flower development. On the other hand, elevated levels of P in soils due to overuse of P-based fertilizers can cause eutrophication of fresh water bodies. Phosphorus levels are affected by several processes including weathering and immobilization, which degrade P levels in soil or make them inaccessible to plants. Run-off and leaching are two water-mediated processes that also decrease available amounts of soil P and are a major problem in the maintenance of phosphorus in agriculture (Hyland et al 2005).

However, mineralization or solubilization can increase available P levels through the microbial conversion of inorganic P to plant-accessible orthophosphates such as HPO_4^{2-} or $H_3PO_4^-$ (Hyland *et al* 2005). This may occur through the production of organic acids and chelated oxoacids found in the soil, including malate, citrate, and gluconate. It has been found that increased levels of glucose and decreased levels of N can result in increased amounts of soluble P, and media with a 2:1 ratio of glucose and $MgCl_2$ gave some of the best rates of solulbilization *in vitro*, indicating that several factors may affect this process in soil systems (Nautiyal 1998). Another indication of phosphate solubilization is decrease in pH often due the production of acidic byproducts by PGPRs (Fankem et al 2006). These organic acids create a "halo" around the organism, which is particularly helpful for their identification as many of these PGPRs are only suspected and cannot be cultured (Nautiyal 1998).

Current Research

In the Sahel of West Africa there are two dominant local shrubs (*Guiera senegalensis* and *Piliostigma reticulatum*) that are found in farmers' fields and can co-exist with crops. These shrubs re-grow after every cropping season but, unfortunately, they are now routinely coppiced and burned in the spring. Even though these shrubs are found in rather low densities, research

has repeatedly shown that their rhizospheres provide nutrients, stabilize the soil biomass, and help maintain water content during the dry season (Dossa et al 2008). The microbial biomass in the soil immediately beneath a shrub was largely maintained, even during ~9 months without rain, whereas there were dramatic reductions in the microbial biomass soil from outside the shrub canopies (Diedhiou-Sall et al 2009). The shrubs do not compete with crops for water, and they help recharge groundwater during the rainy season (Kizito et al 2006). They can also do hydraulic lifting (HL) of water from wet sub-surface regions to the surface soil (Kizito et al 2012). Agronomic research has shown that these shrubs can dramatically increase crop yields (Dossa et al 2008, 2012). This positive response could be due to PGPR that are harbored by shrubs in the rhizosphere and bulk soil beneath the shrubs. However, this is entirely uninvestigated. The purpose of this experiment is to grow these isolates in pure cultures, genotype them using RFLP, and use the genotypes to inoculate millet plants to observe their possible effects on the plants' growth. Based on past research on the roles of PGPRs in plant systems, we predict that certain genotypes of Bacillus will have a positive effect on the final height and biomass of the millet plants.

MATERIALS AND METHODS

The research was done in the Peanut Basin of Senegal in West Africa, which is semiarid and has most of the precipitation distributed from July to October, generally as intense short-duration showers. Millet and peanut (*Arachis hypogaea* L.) are the two major crops. The experimental site (Keur Matar Arame) is in the northern region of the Peanut Basin (14°45' N, 16°51' W, and 43 m above sea level), with mean annual precipitation of 450 mm and temperatures ranging from 20°C in December–January to 33°C in April–June. The soil (Rubic arenosol) (FAO, 2006), known locally as Dior 95% sand, mainly originates from aeolian deposits

and has no distinct horizonation. The topsoil (0–10 cm) has organic matter and total N contents of 0.35 and 0.02% respectively, P content of 95 mg kg^{-1}, and mean pH (water) of 5.5. In this region *G. senegalensis* is the dominant shrub in farmers' fields.

In the July of 2012, six shrubs were randomly selected from each of fifteen plots. From each shrub, two millet plants were selected from within the shrub canopy (1 m away) and two shrubs were selected from more than 5 m away. The relative locations for each sample were recorded. Rhizosphere soil was obtained from each millet plant by gently excavating around and underneath the roots. The intact roots were lifted with soil adhering and gently shaken. The soil remaining was stripped off of the roots and defined as the rhizosphere soil for this experiment. One gram of soil was used to isolate *Bacillus spp*. This was done by vortexing the rhizosphere soil with sterile water and media and applying a heat shock to give twenty-four samples. These samples were plated on Tryptic Soy Agar (TSA) for outgrowth and then moved to glycerol stocks in 96-well plates for storage at 4° C with each colony occupying a separate well. In total, there were twenty-four wells for each of the twenty-four samples.

The samples of *Bacillus* were re-cultured from the glycerol stocks from 2012. The samples were genotyped using Amplified Ribosomal DNA Restriction Digestion (ARDRA), a variation of RFLP that relies only on the amplification of the 16s ribosomal DNA. After an outgrowth step, the DNA from each sample was extracted using a freeze-thaw method involving 3 cycles of freezing the samples at -80° C and thawing them at 94 °C. Prior to the freeze-thaw cycles, 90 uL of 1/5 PCR buffer and sterile water solution was added to 10 uL of *Bacillus* cell culture in fresh 96 well PCR plates with one sample per well coordinating to the placement of the samples in the first set of cultures for the sake of organization. The samples were stored at 4° C for future use. PCR was then conducted to amplify the 16s region of the DNA using a solution

of 5 uL1/5 5x PCR buffer, 1.8 uL 25 mM $MgCl_2$, 2.5 uL 2mM dNTPs, 13.4 uL sterile water, 0.2 uL each of 8F (100 pmol uL^{-1}) and 1492R (100pmol/ul) buffers, 0.04 RNAse A (25mg*ml^{-1}), 0.3 GoTaq Flexi (5 U*uL^{-1}) and 2.0 ul of template DNA for each reaction in fresh 96 well plates. The plates were incubated in a thermocycler according to the following protocol:

1. 5 min 95°C
2. 1 min 94°C
3. 45 sec 54°C
4. 60 sec 70°C
5. Go to 2 x 27
6. 8 min 70°C

The plates were stored at 4°C until for later use.

Success of the PCR amplification was confirmed using a 1.5% Agarose gel electrophoresis and 4 uL of the PCR product and 1 uL of loading dye per well. 4 uL of a 100 base pair ladder was also added. The gel was run at 120 Volts for 30 minutes, stained in Ethidium Bromide (EtBr) for 15 minutes, and destained in water for another 15 minutes. At this point, the gel was observed and photographed under ultraviolet light with GELDOC software for confirmation of the 16s gene amplification and presence of viable DNA in the sample.

If the amplification was successful, restriction digestion was then performed on each of the samples using a solution of 0.5 uL 10x Buffer B, 6.0 uL sterile water, 0.3 ul MspI (10u*uL^{-1}), and 3.5 uL PCR product per reaction in fresh 96 well PCR plates. The restrictive enzyme MspI is designed to cleave the first cystine from the protein with the sequence CCGG. The plates were placed in a thermocycler to incubate for four hours at 65°C and 20 minutes at 37°C. For visualization, the digested DNA was run through a 1.5% agarose gel following the same procedure as described for PCR confirmation above. Pictures of each gel electrophoresis were used to visually compare the samples to each other and to the 100 bp ladder. Groups were made

of samples with the same band pattern and therefore the same or similar genotype. There were eleven different groups and they were used for inoculation of the millet plants. This grouping was revised after the experiment was concluded to include only nine. Further restriction digestion was conducted on the assigned groups to ensure correct genotyping.

Fifty-two millet plants were grown in separate pots in the sandy soil (Rubic arenosol) commonly known as Dior 95% sand from the Keur Matar region. The soil was collected at 5 m from any other shrub and all plant debris were removed before planting to limit any effects the un-inoculated soil may have on the experiment. The soil was not sterilized to best mimic the conditions of the millet plant and microorganism system in farmers' fields. Five millet seeds were added to each pot, which were later thinned to allow the growth of one plant per pot. The plants were grown in a greenhouse in a completely random grid. The randomization of the planting pattern ensured that each group received roughly the same amounts of sunlight, rainwater, and the potential effects of other plants. Plants were watered and their height measured twice a week. When each plant was five to ten centimeters tall, they were inoculated with one sample from each genotype of *Bacillus*.

Prior to inoculation, one sample from each group was selected for outgrowth using 60 uL of the original colonies and 30 mL of TSB in a 50 mL Falcon tube. These were allowed to incubate at room temperature (about 22 °C) for 2 days to ensure the viability of the cells. If no growth was observed after 1 day, another colony from the original plates was chosen to represent the group and a fresh Falcon tube was used to make another solution following the above protocol. Group06 could no longer be included in the experiment because it contained 2 individuals, and neither grew. To verify the identity and confirm correct grouping of the inoculates, DNA extraction, PCR amplification of the 16s ribosomal DNA, and restriction

digestion were conducted using the same techniques listed above.

Millet roots were inoculated with 5 mL of each inoculate poured over the roots of four separate millet plants. Again, group 6 was not included. Two controls were included in the green house experiment; these were 5 mL TSB and 5 mL sterile water. The plants were grown for one month and their height measured twice weekly. The heights of each group were averaged to allow to easier use of the group results. . At the end of the month, the millet plants were uprooted and weighed before and after drying to obtain the wet and dry biomass. Again these results were averaged for each group and later used for statistical analyses.

At this point, samples of rhizosphere soil were taken to check that the inoculate culture of bacteria had survived. After the biomass was obtained, the roots from the groups with the highest average biomass (groups 2, 4, 8, 10, and 11) were removed with a scalpel, weighed, and recorded. Each was then placed in a 5 ml test tube containing 5 – 7 mL sterile water and vortexed for 15 seconds four times to remove the dirt and microorganism from the root. From each test tube, 1 ml of the water-soil suspension was placed in an Eppendorf tube and allowed to incubate in a water bath at 65 °C for 15 minutes. The samples were cooled to about 40 °C, and each sample was then spread on a 1/10 TSA plate for outgrowth at room temperature.

After two days, most of the colonies on the plates were smooth, round, opaque, and white; they resembled colonies typical of *Bacillus*. Three colonies per plate were removed with a sterile toothpick and placed in individual 96-well plates containing 200 ul of 1/10 TSB solution. The plates were allowed to incubate at 45 °C overnight for further growth. These colonies were then used for DNA extraction, PCR, and restriction digestion, as described previously to confirm the genotypes of the remaining *Bacillus* and to compare the genotypes to those of the inoculates. 10 ul of the remaining cells were transferred to fresh 1/10 TSB and outgrowth was again allowed

over night at 45 °C. The resulting cultures are now stored in 1/5 TSB + 20% glycerol stocks at -80°C for future use.

RESULTS & DISCUSSION

From the genotyping portion of this project, 11 distinct banding patterns were found in combinations ranging from 50bp to 350 bp (Image: Figure 6) from about 100 individuals. The groups contain varying numbers of individuals and affected millet plant growth differently. It can be observed that Group08 and Group04 appear have a greater total average height than the rest, but there was no statistically significant ($P < 0.1$) treatment effect (Figure 1). Although mean values were substantially different, numerically in some cases, there was also high variability, which reduced the potential to detect statistically a significant treatment effect.

However, pair-wise T tests of each inoculate treatment with the water and bacterial growth medium controls did detect some significant differences. These are graphically represented in Figures 2, 3, and 4. It was found plants inoculated with *Bacillus* from Group04 showed statistically significant ($P < 0.1$) increase total average final height, total biomass, and belowground dry biomass than the control groups. This indicates that further research could be conducted on any of these inoculates, but particularly on Group04. Group03 was observed to significantly affect the height of millet plants, but as this group of plants has the lowest final average height, it is possible that the inoculants from this group negatively impact the growth of millet. Group11 is also observed to significantly increase the above ground biomass of millet, and Group08 is observed to significantly increase the belowground biomass (Figures 3&4).

Further research is needed to identify the species of Group04 and Group08. They should then be tested to determine the PGPR properties they possess. Following this would be a field test, in which they would be inoculated onto millet and the plants allowed to mature fully.

The increase in growth observed for some *Bacillus* inoculates could be due to one or more plant growth promoting properties, such as increasing the availability of nutrients, production of phytohormones, induction of systemic resistance, or antibiotic properties of the organisms. For example, *Bacillus* has also been a known nitrogen (N) fixer and phosphorous (P) solubilizer for the better part of the past century. As early as 1908, researchers have observed the effects of *B. polyxyma* strains on the increased presence of N in crop soils (Hino, Wilson 1957). Since the Sahelien soil is characteristically low in P, biological inputs in the form of *G. senegalensis* and the microbial community it harbors potentially provide a cost-effective solution.

Additionally, strains of *Bacillus amyloliquefaciens* FZB24, FZB42, and FZB45 and B. subtilus have been observed to significantly increase crop growth with the production of the phytohormone indole-3-acetic acid (IAA). This increase in plant growth has been observed only when high concentrations of IAA are inoculated into the soil. However, this growth was also observed with the inoculation of any of these microbial strains and a low concentation of IAA, indicating that the microorganisms were capable of producing this phytohormone and stimulating plant growth (Idris et al 2004). *B. subtilis* has even been observed to form biofilms on *Arabidopsis* roots and secrete auxins to improve the host's growth (Kumar et al 2011). Because of the pre-existing bacterial community and the low-dose of IAA input needed, this may be a viable and cost-effective option for farmers. Similar results are shown with other *B. subtillus* strains and the production of other phytohormones such as absisic acid and certain cytokinens on the growth of cabbage.

The biofilm may also as a suppressor of plant pathogens by secreting sufractin (Bais et al 2004). In one experiment, *B. subtillus* RB14, as well as other strains, have been shown to

produce the antibiotics Inturin A, Kanosamine, Zwittermycin, and Suffractin, all of which play a role in pathogen suppression in several crop systems. These chemicals are known antibacterials and antifungals (Asaka and Shodo 1996; Van Loon 2005). The potential for *Bacillus* to produce antibiotics has been observed for over 50 years (Stein 2005). Current research suggests that these properties might go beyond anti-microbial factors; one kind of antibiotic, lantibiotics, may act as a hormone, which may also aid in biofilm formation and quorum sensing as effective killing strategies for pathogens (Stein 2005). In this case, a large microbial community would be very effective in reducing pathogen stress on the plant community. Given the diverse array of just *Bacillus* species gathered from just a few cites, it can be assumed that the use of *G. senegalensis* and its microbial community may provide these benefits acting collectively. This further supports the idea that farmers should leave *G. senegalensis* in their fields to receive the shrubs' full benefits.

It can be observed that individuals from the genus *Bacillus* are inherently able produce antibiotics, phytohormones, or increase nitrogen or phosphorous uptake and tolerate high levels of heat and desiccation. Any of these traits would greatly benefit the farmers in the Sahel where agriculture and anthropologic behavior has severely degraded soil and decreased its productivity (Dossa et al 2012). It can be deduced that Bacillus communities supported by the shrub *Guiera senegalensis* may aid millet growth in the Sahel. Additionally, the use of *Bacillus* and other PGPRs would be effective in this region because they are biologically based. Optimized shrub-crop systems that stimulate PGPR could be a way to increase yields and protect crops from diseases. Farmers currently cannot afford fertilizers and pesticides, so replacing or augmenting the current system with a biologically based one would be of little cost to them. And therefore, PGPRs, and especially *Bacillus spp.,* must be considered as pre-harvest biotic factors that affect

both crop yield and quality through the biocontrol of their host plants.

FIGURES

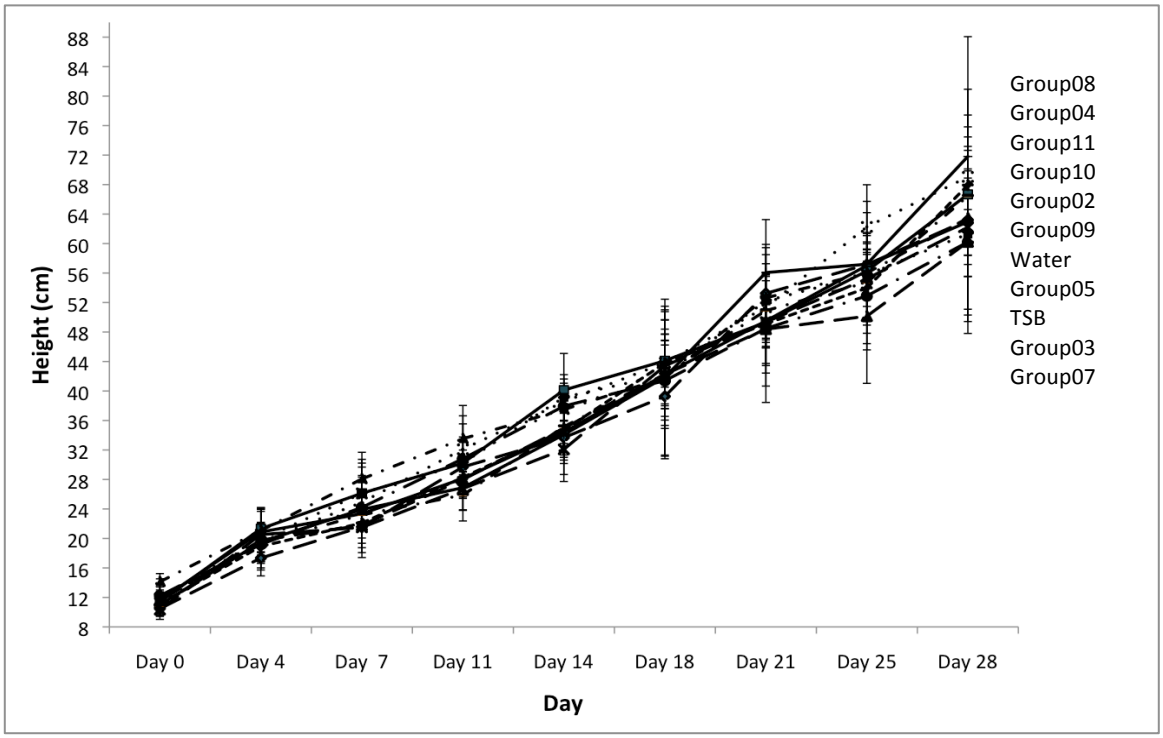

Fig. 1 Plant height in the greenhouse of millet seedlings inoculated with various bacteria isolated from rhizosphere soil of millet plants gown in the presence of the shrub, *Guiera senegalensis*. Legend is arranged in order of height.

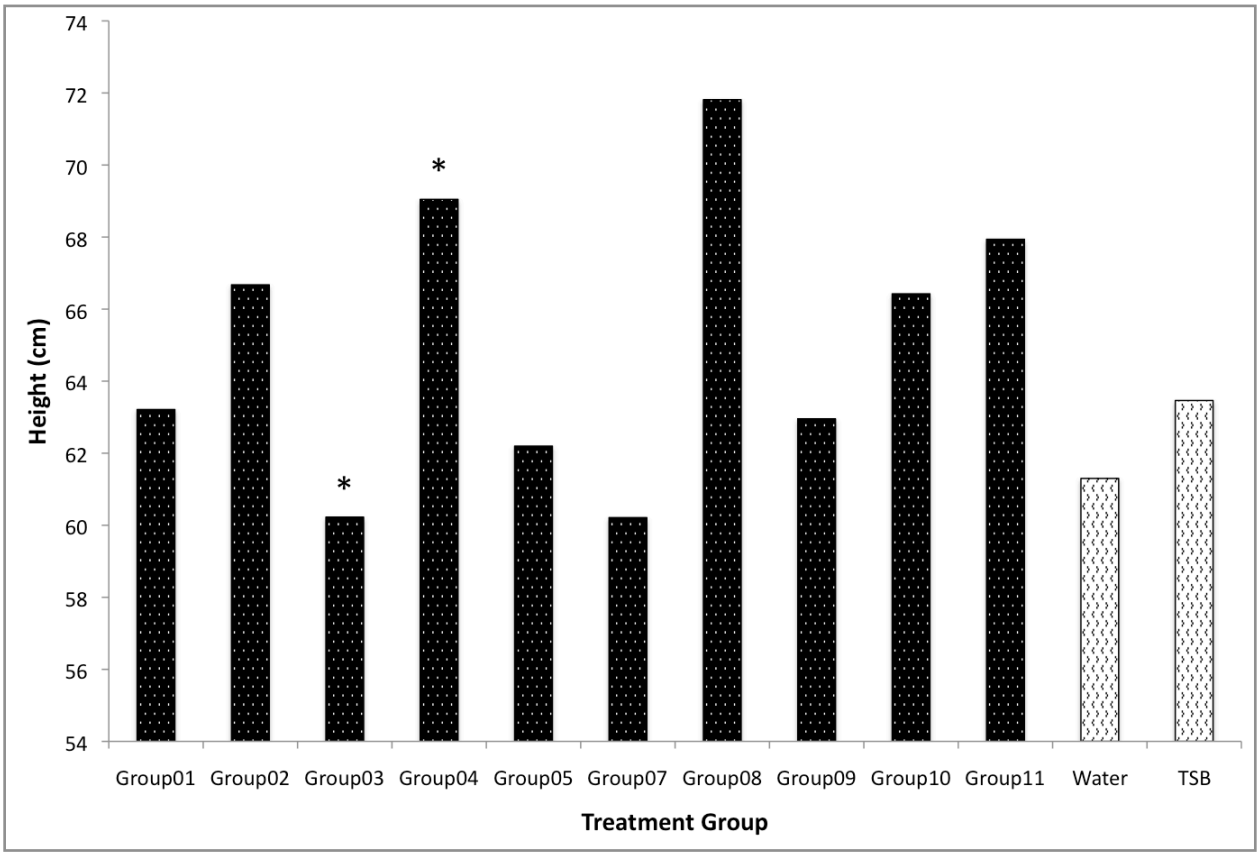

Fig. 2 Average final height per group of inoculated greenhouse millet after 28 days of growth. In a Pairwise Comparison with both controls, Groups 03 and 04 were found to be significant at $P < 0.1$

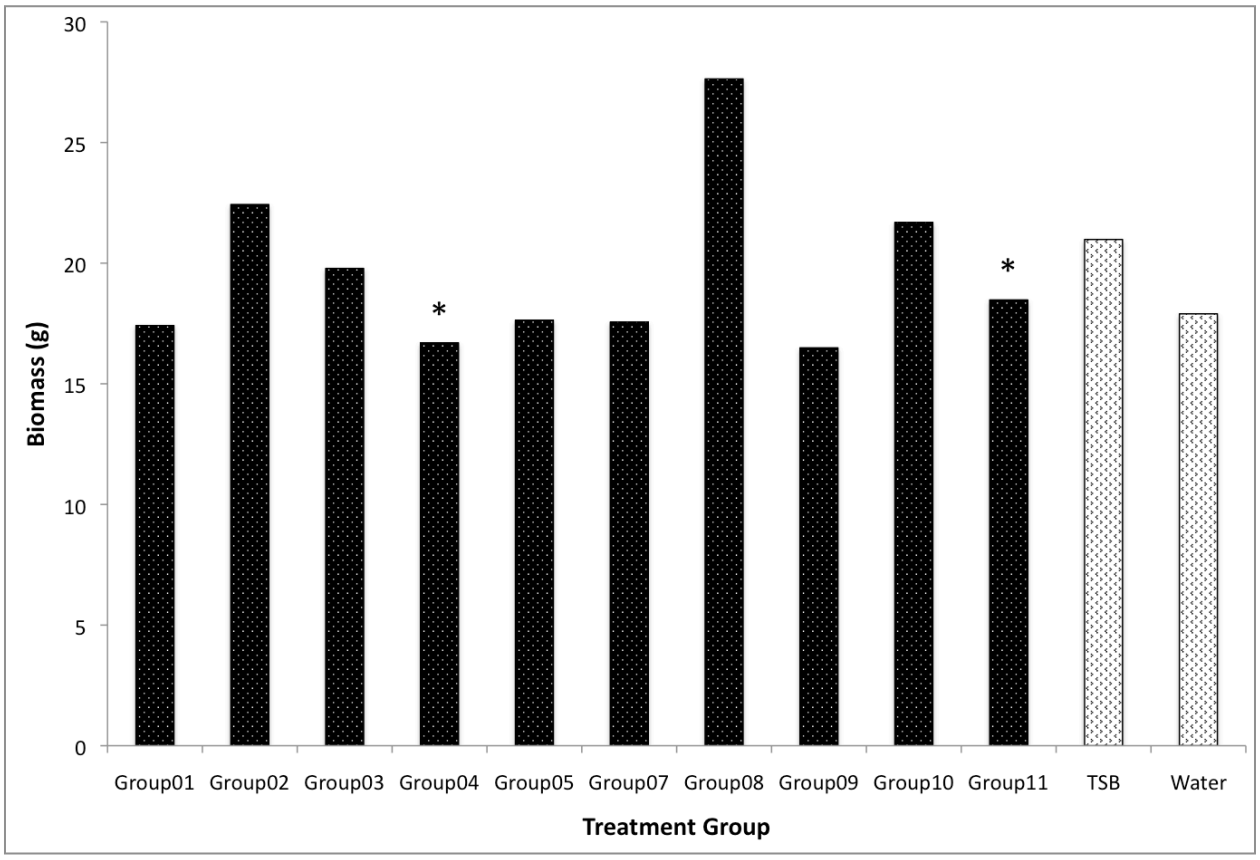

Fig. 3 Average total biomass per group of inoculated greenhouse millet after 28 days of growth. In a Pairwise Comparison with the water control, Groups 04 and 11 were found to be significant at $P < 0.1$

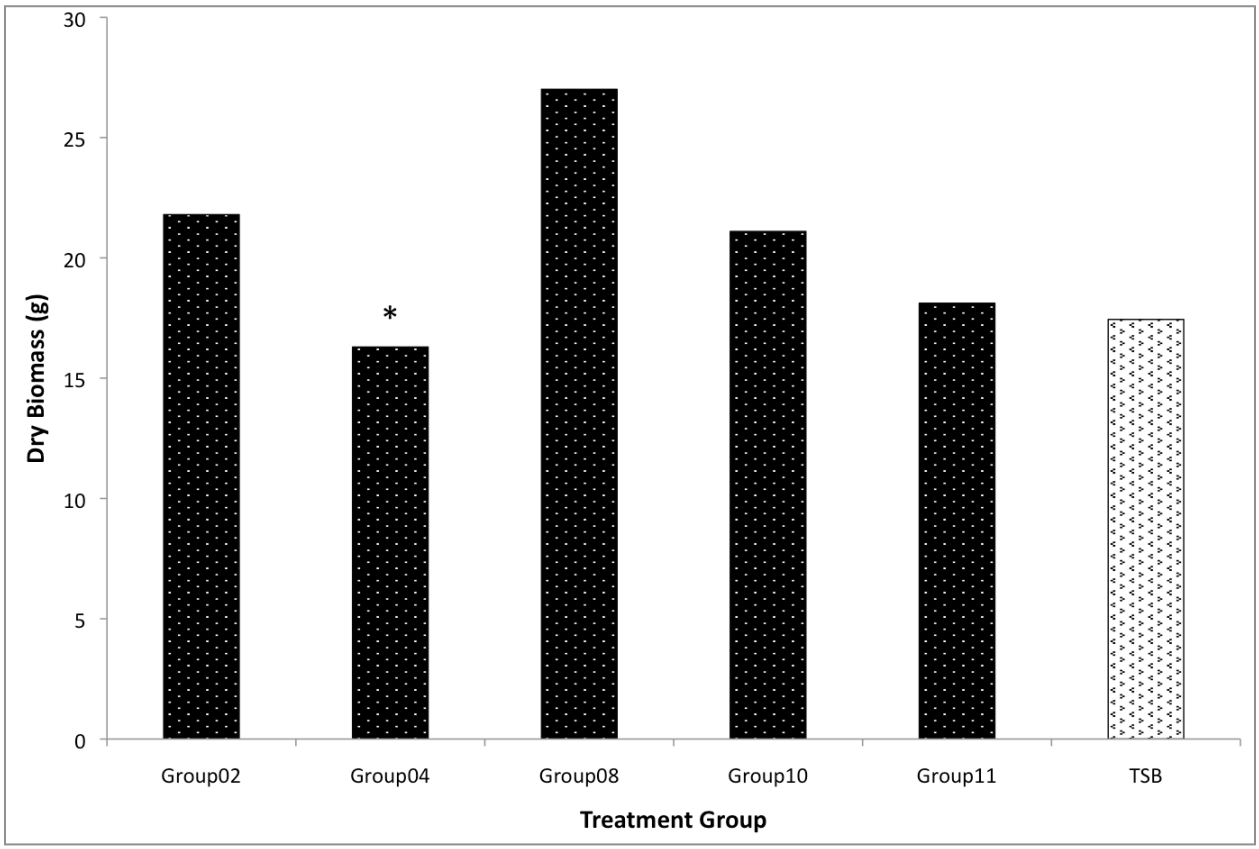

Fig. 4 Average belowground biomass for 6 groups of inoculated greenhouse millet after 28 days of growth. In a Pair-wise Comparison Group08 was found to be significant at $P < 0.1$

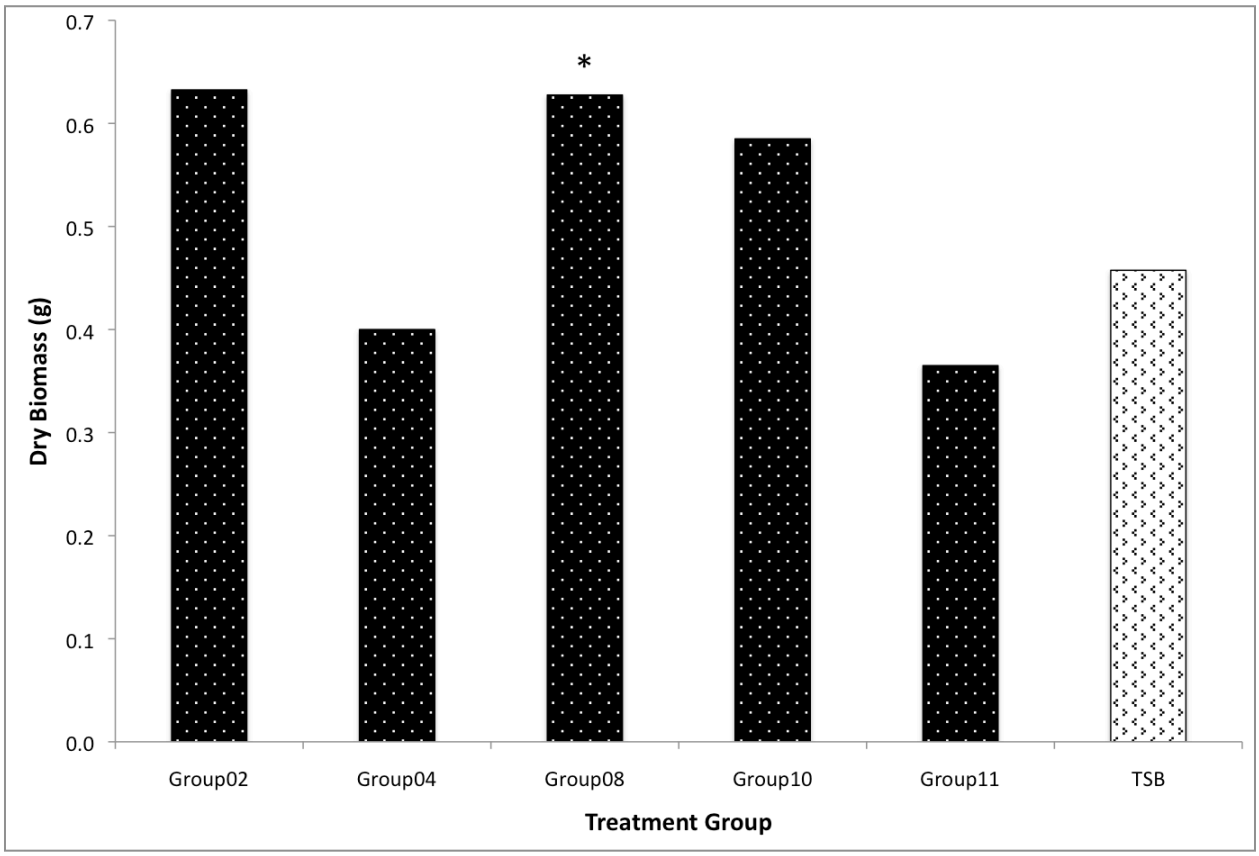

Fig. 5 Average dry aboveground biomass for 6 groups of inoculated greenhouse millet after 28 days of growth. In a Pairwise Comparison Group 04 was found to be significant at $P < 0.1$

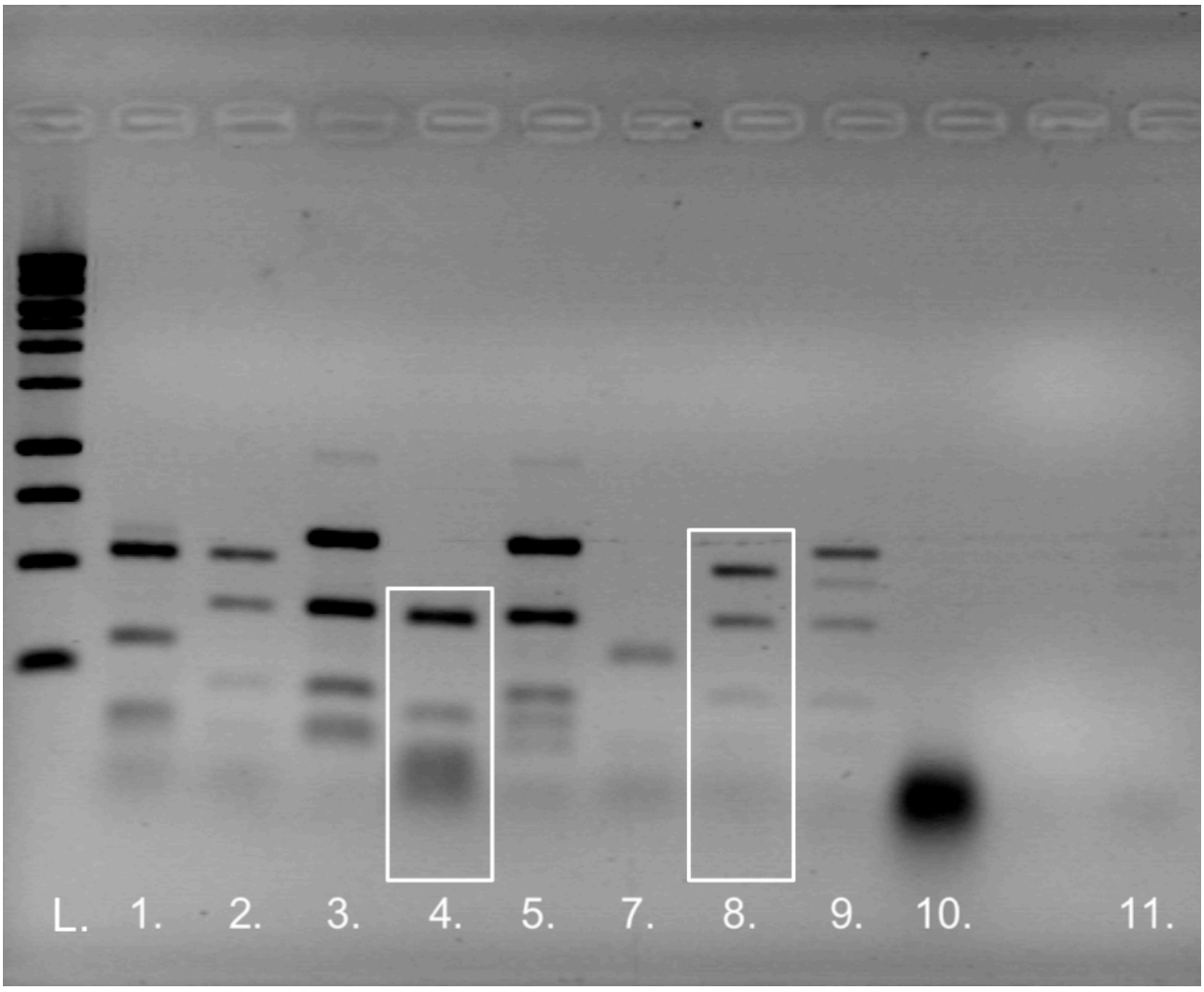

Fig 6. Visualization of 16s DNA after digestion by restriction enzyme MspI. Numbers indicate distinct groups of inoculates. L indicates a 100 bp ladder used to observe band size. Group04 and Group08 are annotated because of their possible future importance.

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